From population to species: morphological and molecular diversity in east African stem borer species of the genus *Manga* Bowden 1956 (Lepidoptera: Noctuidae)

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Abstract. Larvae of noctuid stem borers were collected from wild monocot plants in Eastern Africa, from Ethiopia to Mozambique, and reared to the adult stage. Three species of the African genus *Manga* Bowden 1956 (Lepidoptera: Noctuidae) were found, all restricted to host plants of the family Poaceae. *M. melanodonta* (Hampson) was collected in stems of *Panicum maximum* Jacquin, *Setaria megaphylla* (Steudel) Th. Durand & Schinz and *Setaria plicatilis* (Hochstetter) Engler; *M. nubifera* (Hampson) **stat. rev.**, and *M. fuliginosa* **n. sp.**, were both found only in stems of *P. maximum*. The second species was in the past sunk with *M. melanodonta* as a synonym, but the present study shows its validity. Descriptions are given of the new species as well as of features not yet described of known species (female habitus and male and female genitalia of *M. melanodonta* and M. nubifera) and of the intraspecific morphological variation observed in the male genitalia. Larval morphology and life habits are described. Pictures of the adults and genitalia of the other species of the genus are provided except for *M. bisignata* Laporte that is sunk with *Busseola quadrata* Bowden as a synonym (**n. syn.**). The molecular diversity of the collected species was studied using the mitochondrial gene Cytochrome ***b***. A complex history of successive fragmentation events was revealed. The combination of three forces appeared to have shaped this diversity: the main paleo-climatic events (successive dry and humid periods), the geological barriers, particularly the Rift Valley, and specialization on new host plants. A molecular clock proved to be acceptable for all clades except for the species that first diverged, *Manga fuliginosa*. The dates of the major paleo-climatic events of the last 5 million years appeared to correspond to the observed divergence events when using an evolutionary rate of 1.15% per million years, with a correction for *M. fuliginosa*. Isolation by the Rift Valley favoured diversification in some instances, and the adaptation of *Manga melanodonta* to new host plants enabled the colonization of humid environments. A scenario for the evolution of the group is proposed, from its origin in Austral Africa about 5 million years ago and its northward expansion, until the recent migrations of *Manga nubifera* during the past million years.

Résumé. De la population à l'espèce : Diversité morphologique et moléculaire des foreurs de graminées d'Afrique de l'Est du genre *Manga* Bowden 1956 (Lepidoptera : Noctuidae). Des larves de noctuelles foreuses ont été récoltées dans les tiges de monocotylédones sauvages en Afrique de l'est, de l'Éthiopie au Mozambique, et élevées jusqu'au stade adulte.Trois espèces du genre africain *Manga* Bowden 1956 (Lepidoptera : Noctuidae) ont été trouvées, dans des plantes hôtes appartenant uniquement à la famille des Poaceae. *M. melanodonta* (Hampson) a été récoltée dans des tiges de *Panicum maximum* Jacquin, *Setaria megaphylla* (Steudel) Th. Durand & Schinz et *Setaria plicatilis* (Hochstetter) Engler; *M. nubifera* (Hampson) **stat. rev.** et *M. fuliginosa* **n. sp.**, ont toutes deux été récoltées seulement dans des tiges de *P. maximum*. La deuxième espèce avait été dans le passé mise en synonymie avec *M. melanodonta*, mais la présente étude montre sa validité en tant qu'espèce. Des descriptions sont données de la nouvelle espèce ainsi que de caractères non encore décrits d'espèces connues (habitus femelle et genitalia mâles et femelles de *M. melanodonta* and *M. nubifera*) et de la variabilité intraspécifique observée sur les genitalia mâles. La morphologie et les traits de vie des larves sont décrits. Des photos des adultes et genitalia des autres espèces du genre sont fournies excepté pour l'espèce *M. bisignata* Laporte, qui est mise en synonymie avec *Busseola quadrata* Bowden (**n. syn.**). La diversité moléculaire des espèces récoltées a été étudiée au niveau du gène mitochondrial Cytochrome ***b***. Une histoire complexe, faite d'événements de fragmentation successifs, a été mise en évidence. La combinaison de trois forces semble avoir façonné cette diversité : les événements paléo-climatiques majeurs (succession de périodes sèches et humides), les barrières géologiques, en particulier la Vallée du Rift, et la spécialisation sur de nouvelles plantes-hôtes. Il est apparu que l'hypothèse d'une horloge moléculaire était acceptable pour tous les clades à l'exception de l'espèce ayant divergé le plus anciennement, *Manga fuliginosa*. Les dates des événements paléo-climatiques majeurs des 5 derniers millions d'années sont apparues correspondre aux événements de divergence observés si l'on adopte un taux d'évolution de 1,15% par million d'année, avec une

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correction pour *M. fuliginosa*. L'isolement par la Vallée du Rift a parfois favorisé la diversification, et l'adaptation à de nouvelles plantes-hôtes de *Manga melanodonta* a permis la colonisation de biotopes humides. Un scénario de l'évolution du groupe est proposé, depuis son origine en Afrique australe il y a environ 5 millions d'années et son expansion vers le nord, jusqu'aux récentes migrations de *Manga nubifera* durant le dernier million d'années.

Keywords: Graminaceous stem borer, *Manga*, Molecular clock, Noctuidae, Poaceae.

Noctuid stem borers of graminaceous plants are important pests of crops in Africa where severe yield losses were reported from countries South of Sahara, in Southern (Van Den Berg *et al*. 2001), Western (Moyal 1998), and Eastern Africa (Khan *et al*. 2001; De Groote 2002) as well as from countries North of Sahara (Moyal *et al*. 2002). In order to get a better insight into the ecology and the way of controlling these pests, studies in wild environments have been recommended for a long time (Bowden 1976). Understanding the infestation dynamics, the possibilities of survival of an introduced parasitoid and estimating the risk of shift of a species from wild host plants to cultivated ones need to extend the studies of these insects outside of the crops. A first approach of this kind was carried out in Eastern Africa (Polaszek & Khan 2000) and in Western and Central Africa (Schulthess *et al.* 1997), which led to a better knowledge of wild host plants, mainly for known pests. Studying borer populations in natural landscapes requires the identification of species, which may be difficult, particularly in noctuid borers, because of the great morphological similarity between species and some intra-specific variability (Tams & Bowden 1953; Holloway 1998). So the combined use of morphological and molecular data has been promoted as a powerful tool to get a better insight into the taxonomic relationships in noctuid stem borers (Moyal 2006). Molecular studies can furthermore reveal the recent history of taxa. This approach was used in the present paper, which has the following purposes: (i) to clarify the taxonomy of the small African borer genus *Manga* Bowden 1956 (Lepidoptera: Noctuidae), that includes until now four species (Poole 1989) among which *M. basilinea* Bowden 1956, a pest of pearl millet, *Pennisetum glaucum* (L.) in Western Africa (Bowden 1956; Harris 1962); (ii) to show the morphological variability in the three East African species collected; (iii) to show the phylogenetic relationships and the molecular diversity of these species; (iv) to propose a scenario for the causes and the timing of their evolution.

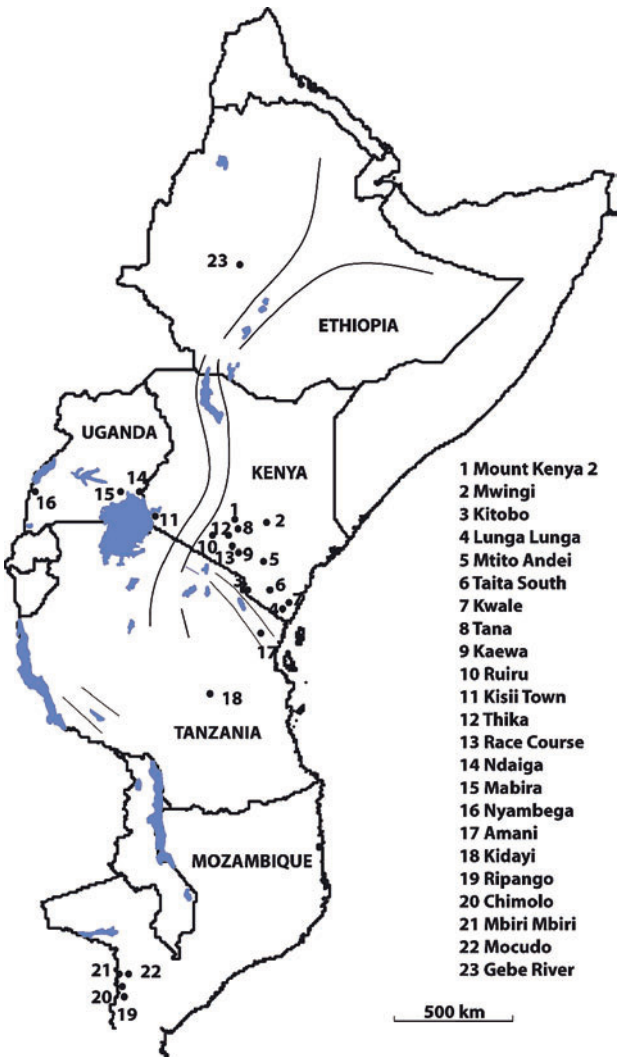

Figure 1
Localities sampled.

Material and Methods

Morphological study

Stem borer larvae were collected from wild and cultivated host plants in several countries of Eastern and Southern Africa: Eritrea, Ethiopia, Uganda, Kenya, Tanzania, Mozambique, Zambia, Rwanda and Zimbabwe. They were then reared on artificial diet (Onyango & Ochieng'Odero 1994) until pupation and emergence of adults. Some adults were kept in absolute alcohol for molecular analyses, others were kept dry and prepared as vouchers for museum collections and also used for molecular studies. Genitalia were dissected after a short stay in boiling potash 10% bath, and then mounted on slides in Euparal. The type of a new species collected is deposited in

the Museum National d'Histoire Naturelle (MNHN) in Paris, France. Figure 1 shows the location of the sampling sites and table 1 lists the precise geographic position of the sites together with relevant host plant records.

Molecular study

Most of the mitochondrial gene Cytochrome *b* (940 out of a total of 1134 nucleotides) was sequenced from 76 specimens. This gene is widely used in molecular studies in vertebrates and proved to be interesting in insects, particularly at the infra generic level (Simmons & Weller 2001).Total DNA was extracted using a Qiagen DNeasy tissue kit (Qiagen GmbH, Germany). The Cytochrome *b* gene was amplified by Polymerase Chain Reaction (PCR) using the successive steps: initial denaturation for 5 min at 92 °C; 39 cycles of denaturation for 1 min at 92 °C, annealing for 1.30 min at 46 °C, extension for 1.30 min at 72 °C; final extension for 5 min at 72 °C. The reaction mixture contained 3 mM $MgCl_2$, 0.4 µM primers, 0.24 µM dNTPs, 2 U of Promega *Taq* polymerase and 100 ng of DNA per 50 µl of reaction mixture. The primers used were CP1 (5'-GATGATGAAATTTTGGATC-3') [modified from Harry *et al.* (1998)] and TRs (5'-TCTATCTTATGTTTTCAAAAG-3') (Simon *et al.* 1994). The PCR product was then purified using the Qiagen QIAquick PCR purification kit (Qiagen GmbH, Germany). Sequencing reactions were carried out using the Sanger dideoxy method (Sanger *et al.* 1977), and finally sequences were run and detected on an ABI 377 automated sequencer. A portion of the mitochondrial gene cox1 (Cytochrome c Oxidase, subunit 1) (894 nt) was also sequenced for a limited number of insects in order to study the possibility of dating the divergence events (see below). The primers used were Ron (5'-GGATCACCTGATATAGCATTCCC-3') and Hobbes (5'-AAATGTTGNGGRAAAAATGTTA-3') (Monteiro & Pierce 2001) and the PCR protocol was the same as for Cytochrome *b*.

Phylogenetic analysis

The obtained sequences were aligned using Multalin (Corpet 1988). The best substitution model was selected with Modeltest ver 3.7 (Posada & Crandall 1998) in combination with Paup 4.0b10 (Swofford 2003) using the Akaike information criterion (Posada & Buckley 2004). The values of the selected parameters were then used to perform the phylogenetic analysis with Phyml software (Guindon & Gascuel 2003). The algorithm used in this software enables a fast estimate of the best phylogenetic tree using the maximum-likelihood principle, thanks to the simultaneous adjustment of tree topology and branch lengths, using the nearest neighbour interchange as branch swapping method. Except the selected parameters, the default options proposed by Phyml were used, with a starting tree built by BIONJ (Gascuel 1997). This analysis was followed by non parametric bootstrapping of 700 replicates. Three species belonging to closely related borer genera were chosen as outgroups: *Busseola fusca* (Fuller 1901), *Sesamia calamistis* Hampson 1910 and *Sciomesa nyei* Fletcher 1961. The phylogenetic tree was then displayed as a graphic in Treeview ver 1.6.6 (Page 1996). Genetic distance and divergence dates were calculated with Mega 3.1 (Kumar *et al*. 2004). Diversity at the population level was studied only in *M. nubifera* (49 sequenced individuals). Haplotype networks were constructed according to the method of Templeton *et al.* (1992) with TCS version 1.18 (Clement *et al*. 2000). Arlequin software (Schneider *et al.* 2000) was used to calculate diversity parameters (gene diversity, pairwise differences and nucleotide diversity) and migrant numbers between populations using the Slatkin's method (1991).

To understand the role of paleo-climatic events on the diversification of the species, it is necessary to be able to date the divergence events. Estimation of the rate of evolution in insects is difficult because of the lack of fossils in many orders. Two studies have attempted to solve this question, using mitochondrial DNA. These two methods were compared to estimate the divergence dates between the observed clades of *Manga*. The first one, proposed by Brower (1994), compared the evolution rate of recent species (whose divergence occurred less than 3.5 million years ago) belonging to several orders, using several methods of calibration of the evolutionary rate (e.g., fossil dating when available and biogeography). Different methods of studying mitochondrial diversity were used, depending on the available data (Restriction enzymes sites and sequences of Cytochrome c Oxidase subunit 1). This study indicated that the evolution rate was rather constant (i.e. a molecular clock is acceptable) whatever the method used and the insect order considered. The evolutionary rate of mitochondrial DNA was then estimated to be 1.1-1.2% per million years, i.e. an average pairwise divergence of 2.3%. The second method was used by Gaunt & Miles (2002) in order to date all the important events of the insect evolution, and then much older divergences. The use of slowly evolving sites was then necessary. Their study, based on cox1 gene, examined the evolution of the second codon position and aminoacids. They showed that a molecular clock was acceptable in the first case but that local molecular clocks had to be fitted for protein evolution. These two methods may result in large differences between estimates of divergence events, for instance an evolution rate about 6 times slower in the second method in the case of Papilionidae (Zakharov *et al.* 2004). Therefore one needs to estimate which method is most appropriate to get a correct estimate of speciation events. The hypothesis of a molecular clock was tested with Mega 3.1, using the relative rate test of Tajima (1993).

Results

Specimens of *Manga* Bowden 1956 were found in five of the surveyed countries: Ethiopia, Kenya, Tanzania, Uganda and Mozambique and in only three host plants: *Panicum maximum* Jacquin 1781, *Setaria megaphylla* (Steudel) Th. Durand & Schinz 1894 and *Setaria plicatilis* (Hochstetter) Engler 1891 (Poaceae). Three species were collected: *Manga melanodonta* (Hampson 1910) and two other species, i.e. one new species (described below as *M. fuliginosa* **n. sp**) and a species corresponding to *M. nubifera* (Hampson 1910) that was however sunk with *M. melanodonta* as synonym by Fletcher (1961). This study showed that it was a true species and its status is therefore re-appreciated. Indeed, the types of both *M. melanodonta* and *M. nubifera*, examined at the Natural History Museum of London, showed slight morphological differences, particularly in the male genitalia (figs. 3a & 5a). These differences, as well as differences in the habitus, were also observed in the numerous specimens collected, showing that both taxa are morphologically distinct. Studies at the molecular level confirmed that

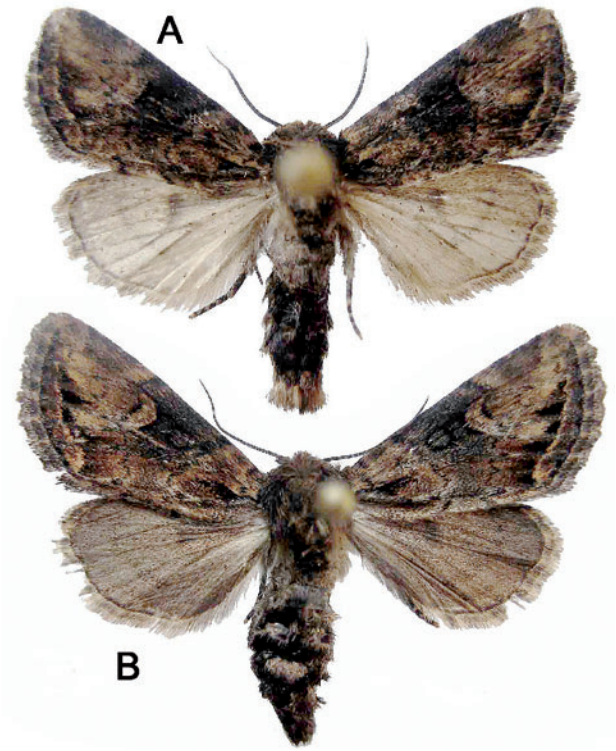

Figure 2
Adults of *Manga melanodonta*. A. Male; B. Female.

both groups diverged long ago. *Manga nubifera* and *M. fuliginosa* were found only in stems of *P. maximum*, whereas *M. melanodonta* was found in the three plants. Sampling in localities where the three borer species were present, e.g. Ripango, showed that all the specimens found in *S. megaphylla* belonged to *M. melanodonta*; moreover the other species were absent in places where only *S. megaphylla* or *S. plicatilis* were present. *M. melanodonta* was found mainly in humid forests such as the Guineo-Congolian vegetation mosaics, whereas *M. nubifera* was located mostly in dry forests or in forest galleries in dry vegetation mosaics (Zambesian miombo and Somalia-Masai mosaics). Too few specimens of *M. fuliginosa* were collected to enable conclusions on its ecological preferences.

Morphological study

Descriptions are given below of the new species, for which only two males were collected. Only the habitus of the male of *M. melanodonta* and *M. nubifera* were known until now and described by Hampson (1910). The male genitalia as well as the females were not known and are described here. Descriptions of larval morphology and ecology, that were unknown until now, are also given.

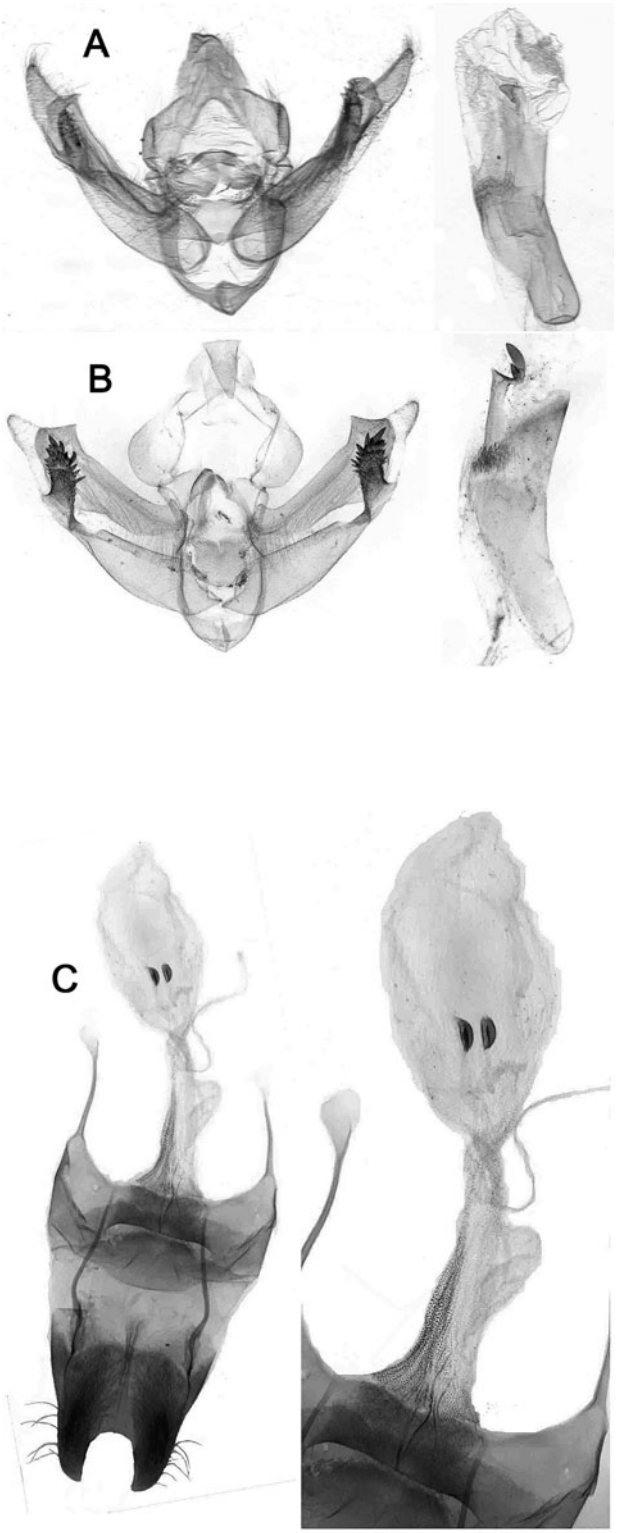

Figure 3
Genitalia of *Manga melanodonta*. A. Male, holotype; B. Male, specimen from Mozambique (Chimolo); C. Female (Uganda, Mabira).

Table 1. Sampling sites and host-plants (S: *Setaria megaphylla* or *S. plicatilis*; P: *Panicum maximum*) of the *Manga* species collected and sequenced.

Country	Site	Latitude	Longitude	*Manga* species	Host-plant
Kenya	Mount Kenya	S 00°43'02"	E 37°16'02"	*M. nubifera*	P
	Mwingi	S 00°33'24"	E 38°03'24"	*M. nubifera*	P
	Kitobo	S 03°15'54"	E 37°22'12"	*M. nubifera*	P
	Lunga Lunga	S 04°19'30"	E 39°04'42"	*M. nubifera*	P
	Mtito Andei	S 02°24'23"	E 38°07'02"	*M. nubifera*	P
	Taita South	S 03°17'51"	E 38°11'12"	*M. nubifera* *M. melanodonta*	P P
	Kwale	S 04°05'18"	E 39°16'11"	*M. nubifera*	P
	Tana	S 00°47'21"	E 37°15'55"	*M. nubifera*	P
	Race Course	S 01°31'33"	E 37°14'38"	*M. melanodonta*	P
	Ruiru	S 01°03'26"	E 36°32'53"	*M. melanodonta*	P
	Kisii Town	S 00°23'55"	E 34°26'23"	*M. melanodonta*	S
	Thicka	S 01°00'46"	E 37°04'17"	*M. melanodonta*	P
	Kaewa	S 01°26'32"	E 37°18'32"	*M. melanodonta*	P
Uganda	Ndaiga	N 00°21'09"	E 34°01'58"	*M. nubifera*	P
	Mabira	N 00°26'27"	E 33°10'21"	*M. melanodonta*	P
	Nyambega	N 00°30'05"	E 30°07'34"	*M. melanodonta*	P
Tanzania	Amani	S 05°03'39"	E 38°24'18"	*M. nubifera*	P
	Kidayi	S 07°20'52"	E 36°28'30"	*M. nubifera*	P
Mozambique	Ripango	S 19°15'46"	E 33°10'37"	*M. nubifera* *M. melanodonta* *M. fuliginosa*	P S P
	Chimolo	S 19°02'43"	E 33°21'27"	*M. melanodonta*	S
	Mbiri Mbiri	S 18°31'01"	E 32°26'09"	*M. melanodonta*	P
	Mocudo	S 18°30'36"	E 32°27'07"	*M. melanodonta*	S
Ethiopia	Gebe River	N 08°08'06"	E 37°20'43"	*M. nubifera*	P

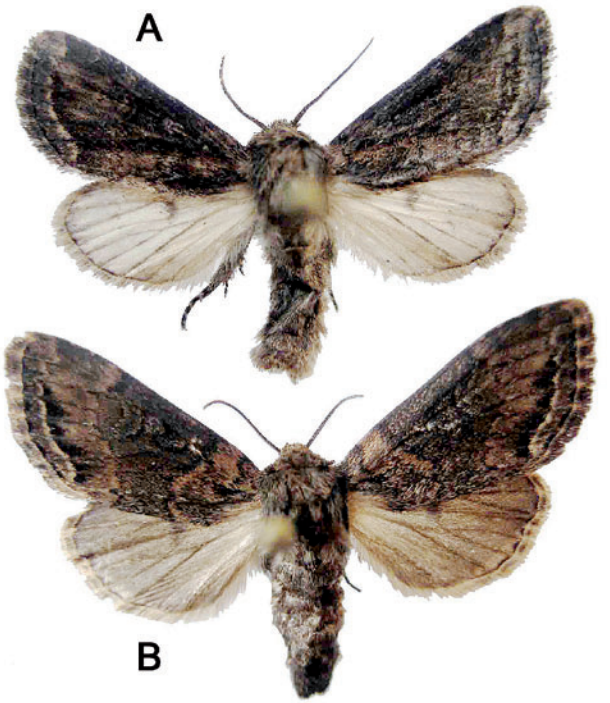

Figure 4
Adults of *Manga nubifera*. A. Male; B. Female.

Manga melanodonta (Hampson 1910)

(Figs 2 & 3)

Male genitalia. Tegumen broadly triangular, with medium-sized rounded peniculi. Vinculum narrow, forming a moderate saccus, like a small bulge of vinculum. Valve narrow, elongate; costa broad and slightly sclerotized with a well-developed ridge-like expansion. Sacculus narrow, broadest at base, gradually tapering distally, without produced clavus; clasper well developed, heavily spinose, straight and of rather constant width from its basis to apex; cucullus broad, fairly triangular with a more or less sharp apex; clasper and cucullus showing some geographic variability (see further); juxta simple, broad, shield-shaped. Uncus short and broad. Aedeagus short, stout, slightly dilated at apex. Apex extending ventrally into a recurved band ending in one or several spines (fig. 3 where the band was everted)**.** Vesica membranous lacking cornuti. Manica membranous with lateral hair tufts.

Female. Similar to the male described in Hampson (1910) except for the ground colour of hindwing which is brown-grey, so that the discal spot is inconspicuous. Ground colour of the wings is generally darker than in the male, rendering the features less easy to distinguish. The female is larger than the male (wingspan: 26-27 mm versus 23-26 mm for the male).

Female genitalia. Both pairs of apophyses long and slender, with little sclerotized spatulate tips. Antrum ovoid, ostium bursae with anterior lip indented and convex in its central part. Sternum A8 slightly sclerotized only anteriorely to ostium bursae. Ductus bursae broad, with a faint punctuated sclerotisation. Bursa copulatrix elongate, elliptic, the length about twice the width, with paired signa; both signa located at approximately one third to one half of the bursa length from the junction with ductus; signa elongate, elliptic or drop-shaped, the length about twice the width, and divided into two parts by a longitudinal midline, generally well conspicuous. Ductus seminalis from junction of bursa and ductus bursae. Ovipositor lobes narrowly triangular, with the apex slightly curved inwards, bearing numerous long setae on the dorsal surface and shorter and sparser setae on the ventral side. The apex of the lobes bears several stout setae (in most cases between five and ten).

Material examined. Cf. Tab. 1.

Manga nubifera (Hampson 1910) stat. rev.

(Figs 4 & 5)

Male genitalia. Similar to *M. melanodonta* but with the following differences: Saccus better defined, narrower and with a roughly quadrangular shape. Valvae with costal sclerotization narrower; cucullus long, slender and with rounded apex; clasper heavily spinose, thick in its basal part, and terminating with sharp narrowing apex and angled towards the cucullus.

Female. Similar to the male described by Hampson (1910) except for the ground colour of the hindwing which is brown-grey, and where the discal spot is inconspicuous. The female is on average larger than the male (wingspan: 25-29 mm versus 24-28 mm for the male).

Female genitalia. As for *M. melanodonta*.

Material examined. Cf. Tab. 1.

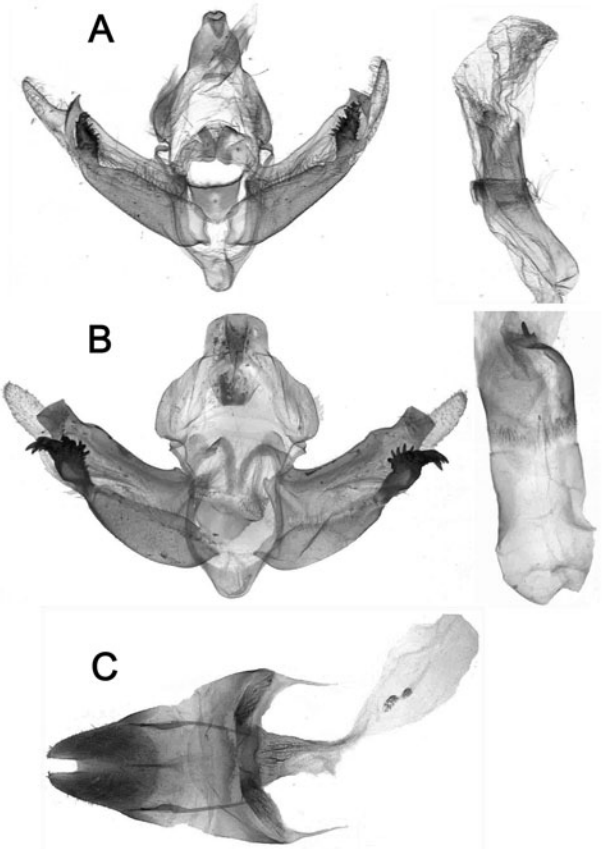

Figure 5
Genitalia of *Manga nubifera*. A. male, holotype; B. male, specimen from Ethiopia (Gebe River); C. female (Kenya, Kitobo).

Manga fuliginosa n. sp.

(Fig. 6)

Material examined. Holotype: ♂, Mozambique, Ripango (19°15'46" S, 33°10'37" E), IV.2005, ex larva (in stem of *P. maximum*), B. Le Rü leg., gen. prep. MP1, MNHN, Paris.

Paratype. ♂, same data as above, gen. prep. MP2, in coll. P. Moyal.

Male. Antennae filiform with the dorsal surface covered with white scales. Head, palpi and thorax dark brown mixed with yellow brown; tarsi brown with slight pale rings; abdomen grey. Fore wing grey before antemedial with a short black basal streak; dark-grey between antemedial and postmedial lines except for a grey-brownish median streak. Claviform and orbicular absent; reniform faint. Subterminal line faint; subapical black spot fairly diffuse. Colouration between postmedial and subterminal lines mainly grey mixed with fuscous brown in the anal half, and dark grey between the subterminal and terminal lines. Hindwing pale grey with a faint discal spot and postmedial line. Wingspan: 20 mm

Male genitalia. Similar to *M. melanodonta* but with the following differences: saccus like a broad bulge of vinculum. Valvae with broad and triangular cucullus with a rounded

Figure 6
Manga fuliginosa. A. Male adult; B. Male genitalia.

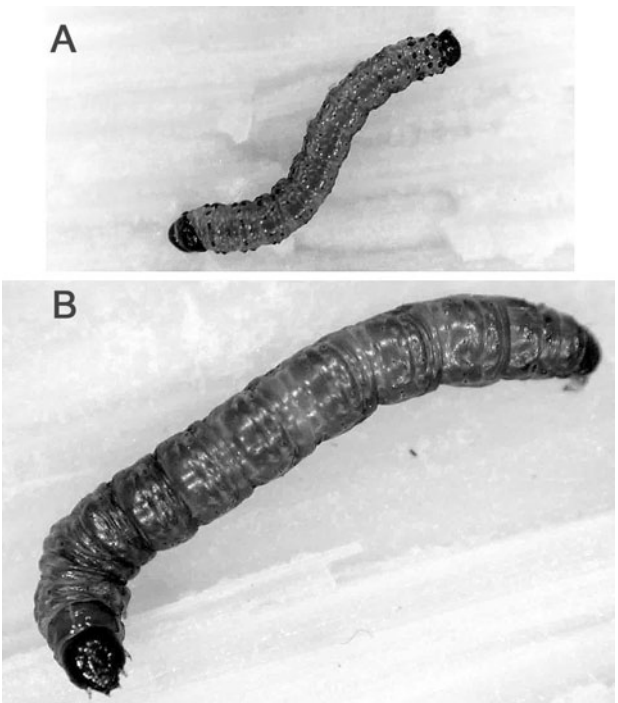

Figure 7
Larva of *Manga nubifera*. A. Young; B. Mature.

apex and strongly convex sacculus; clasper curved inwards, little spinose, with only some strong spines at the apex. The sclerotized band at the apex of the aedeagus with curved sharp spines.

Female. Unknown.

Larval morphology (fig. 7) and preimaginal life habits. The larvae of the three species were morphologically indistinguishable. Their main traits varied from the first to late instars. Young larva, from the first to the third instar (fig. 7A): head smooth and black, prothoracic shield, pinacula and anal plate dark brown, body with variable ground colour from white to pale pink with four characteristic black spots, two dorsal and two lateral, on each of the last three abdominal segments.

Mature larva (fig. 7B): length 30-35 mm, width 3.5 mm. Head smooth and brown, prothoracic shield pale-brown; body with ground colour dark grey, dorsally suffused with pale grey, pinacula and anal plate dark-brown.

Like other noctuid stem borers, eggs are laid between the leaf sheath and stem in batches of up to 50 eggs at the bottom of the spike. After hatching, larvae do not feed on leaves and penetrate the spike where they remain gregarious during the first two-three instars. More than 50-60 second instar larvae are commonly found in the same spike. Usually the apical part of the spike dries and, under dry weather conditions, the second and the third instar larvae remain quiescent until the beginning of the next rainy season, when they become active and disperse onto the stems and leaves of neighbouring plants.

Molecular study

Phylogenetic analysis, geographic distribution and host-plant

The sequences were submitted to GenBank (accession numbers DQ536363-DQ536396 and DQ628527-DQ628539). The best model of DNA evolution selected was HKY (Hasegawa *et al*. 1985) with a proportion of invariant sites of 0.64 and a Gamma distribution with a shape parameter of 1.53. The maximum likelihood phylogenetic tree obtained (fig. 8) shows different clades strongly supported by high bootstrap values. The first divergence event resulted in two species, *M. fuliginosa* and the ancestor of *M. melanodonta* and *M. nubifera*, which divided then into these two species. *M. nubifera* divided then into two clades, one of them with a geographic distribution limited to the Kenyan eastern part of the Rift Valley (MN1) (fig. 9), the second one (MN2) stretching from Ethiopia to Mozambique, limited to the west of the Rift Valley in Ethiopia, Uganda and Kenya, except, in the latter country, in the southern locality of Kitobo Forest. The population of Uganda (Ndaiga) appeared to be the sister group of all other populations in MN2. In *M. melanodonta*, a first division resulted in a Mozambique group (MOZ) and the ancestor of a northern group (Uganda-Kenya), which then split into two clades, one in Uganda (U) and one in Kenya

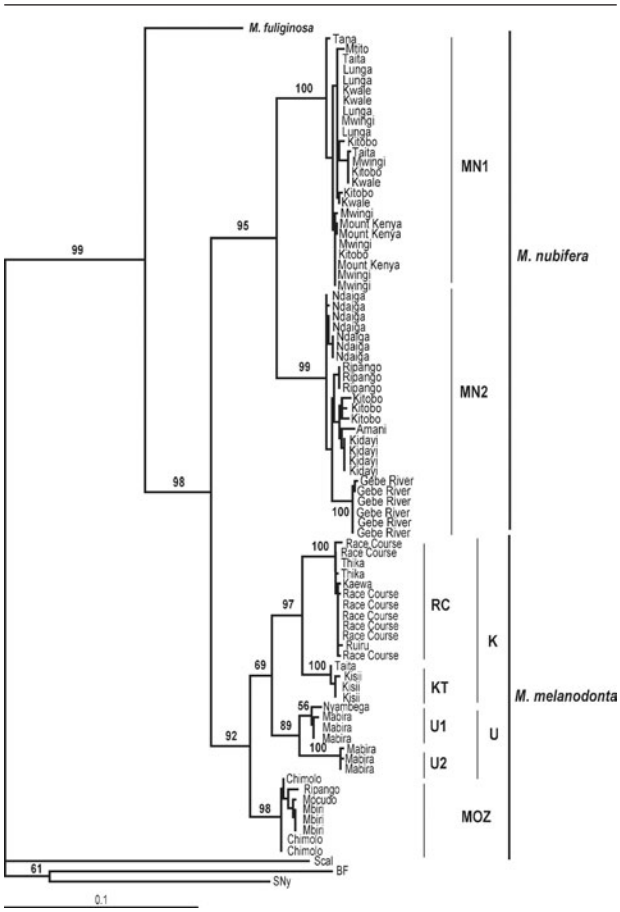

Figure 8
ML phylogenetic tree with the bootstrap values over 50%. Outgroups: SNy: *Sciomesa nyei*, BF: *Busseola fusca*; Scal: *Sesamia calamistis.*

(K) (fig.10). Within these clades a further division occurred: in the Kenyan group, two clades diverged, RC (for Race Course) and KT (for Kissii Town) east and west of the Rift Valley, respectively. In Uganda two different clades (U1 and U2) were found in the same localities. Inside these clades, the genetic distance between specimens was low, between 0 and 0.5%, typical of population variations. The genetic distances between clades were similar in several cases: 0.0241 (std err 0.0045) between U1 and U2, 0.0290 (std err 0.0054) between RC and KT; 0.0498 (std err 0.0064) between MN1 and MN2; and 0.04325 (std err 0.0062) between U and K. The p-distance between both species was 0.06635 (std err 0.0070).

Dating the divergence events

The method used by Gaunt & Miles (2002) appeared to be difficult to use in the present study. Indeed, no variation was observed between *Manga* species for the

Figure 9
Geographic distribution of the clades of *Manga nubifera*.

Figure 10
Geographic distribution of the clades of *Manga melanodonta*.

second codon in the sequenced part of the cox1 gene. Also, among the 298 amino acids, only one mutation was observed, which separates *M. nubifera* from the other *Manga* species. *M. fuliginosa*, which is the sister group of the other two *Manga* species, did not differ from *M. melanodonta* for the amino acid composition. Dating divergence using Brower's method, assuming an evolutionary rate of 1.15% by million years, was done for the different nodes. Most of the clades have separated distribution areas, which suggests that their evolution was independent. The results of the Tajima's relative rate test indicated that the hypothesis of a molecular clock could not be rejected in all cases except for *M. fuliginosa*. The plot of transitions versus transversions (fig. 11) showed indeed an increase from 1 to 12 transversions, but beyond the transition level seems rather constant, which indicates an apparent saturation that corresponds to the comparisons with *M. fuliginosa*. The regression line fitted was highly significant for the first part (without *M. fuliginosa*) ($R^2 = 0.592$; $F = 736.0$, $df = 1$ and 507) whereas there was no significant relationship and the slope was not different from zero for the distances including *M. fuliginosa* ($R^2 = 0.0024$; $F = 0.0081$ $df = 1$ and 33). Since the hypothesis of a molecular clock was acceptable for all the clades for which the regression line was significant, this linear relationship between transitions and transversions can be considered as the expression of this constant evolutionary rate. The actual divergence between *M. fuliginosa* and the other

Table 2. Datation of divergence events assuming an evolutionary rate of 1.15% per million year (MF = *M. fuliginosa*; MM = *M. melanodonta*; MN = *M. nubifera*), with correction for MF (see text).

Node	Divergence date in Million years
MF vs Ancestor MM-MN	4.61
MM vs MN	2.91
MN1 vs MN2	1.99
MOZ vs U vs K	1.86
RC vs KT	1.34
U1 vs U2	1.02

clades if no saturation had occurred should then be correctly estimated through the extrapolation of the regression line. A corrected pairwise distance between *M. fuliginosa* and the other clades was then estimated for the average transversion distance observed (17.34). This yielded a total pairwise distance of 100.07 mutations, and then an estimated divergence date of 4.61 million years (Myr) (tab. 2).

Intraspecific diversity in *M. nubifera*

The intraspecific sampling of *M. nubifera* enabled a first comparison between the population diversity of two clades differing in their distribution area: the first one, limited to Kenya east of the Rift Valley (MN1), and the second one largely distributed from Ethiopia to Mozambique, and mainly west of the Rift Valley (MN2). The haplotype network of MN1 is characterized by closely related haplotypes (fig. 12A) and the presence of several different haplotypes in the localities. The haplotype network of MN2 (fig. 12B) is much more extended, with particularly the population of Ethiopia (Gebe River) very distant from the others (12 mutations from the closest populations) and also an haplotype from Tanzania (Amani) rather far from the centre of the network. This centre includes populations that were geographically close (Uganda, Centre Tanzania and South Kenya) but also the very geographically distant population of Mozambique (2257 km between Ndaiga and Ripango vs 934 km between Ndaiga and Gebe River).

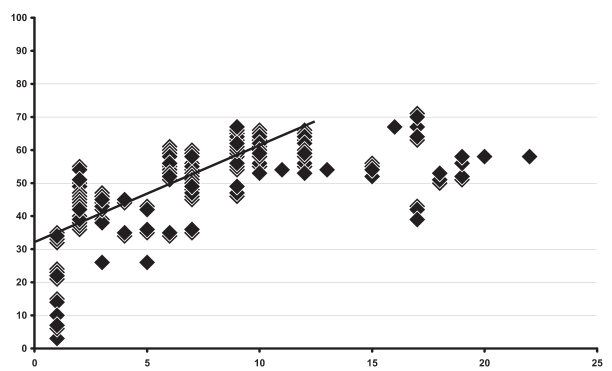

Figure 11
Plot of the pairwise number of transitions versus the pairwise number of transversions, with the significant regression line for the data without *Manga fuliginosa* (see text).

The gene diversity is high and similar in both clades (tab. 3), but MN2 is much more diverse at the nucleotide level, due particularly to the Ethiopian population. The gene flow between populations appear to be much higher in MN1 than in MN2, where all populations seem to be isolated (Slatkin's M value far less than 1). However even in MN1, gene flow can be very reduced: populations of South East Kenya (Kitobo, Taita, Mtito) have a very high gene flow with populations of coastal Kenya (Lunga, Kwale) (Slatkin's M value = infinite) (207 km between Kitobo and Lunga) but much less with Northern populations (Mount Kenya 2, Tana, Mwingi) (Slatkin's M value = 1.96) (304 km between Kitobo and Mount Kenya 2), whereas the gene flow is still much more reduced between northern and southern Kenya (Slatkin's M value = 0.84) (486 km between Mount Kenya 2 and Lunga).

Discussion

The three species are morphologically very similar but can however be distinguished by their habitus. To the naked eye *M. nubifera* appears in most cases dark, without visible elements of pattern, whereas in *M. melanodonta* the distal upper end of the forewing, including the reniform, appears like a large rounded pale spot. *M. fuliginosa* is much smaller than the other two species and has no pale spot but is neither so homogeneously coloured nor as dark as *M. nubifera*. Its hind wing is also much greyer than in the other two species, in which this is nearly white.

Some intraspecific variation was detected in the male genitalia (figs. 13 & 14), particularly with regard to the number and shape of spines at the extremity of the sclerotized band of the aedeagus. The number of spines was variable within species (between one and four spines) and did not enable the distinction of the different species. Some differences in shape were observed, but they were not species-characteristic: the spines were curved in *M. melanodonta* but only in populations from Mozambique and Uganda, and straight in the Kenyan population (Race Course) as it was the case of *M. nubifera*. In *M. fuliginosa* the spines were curved like in the first two populations of *M.*

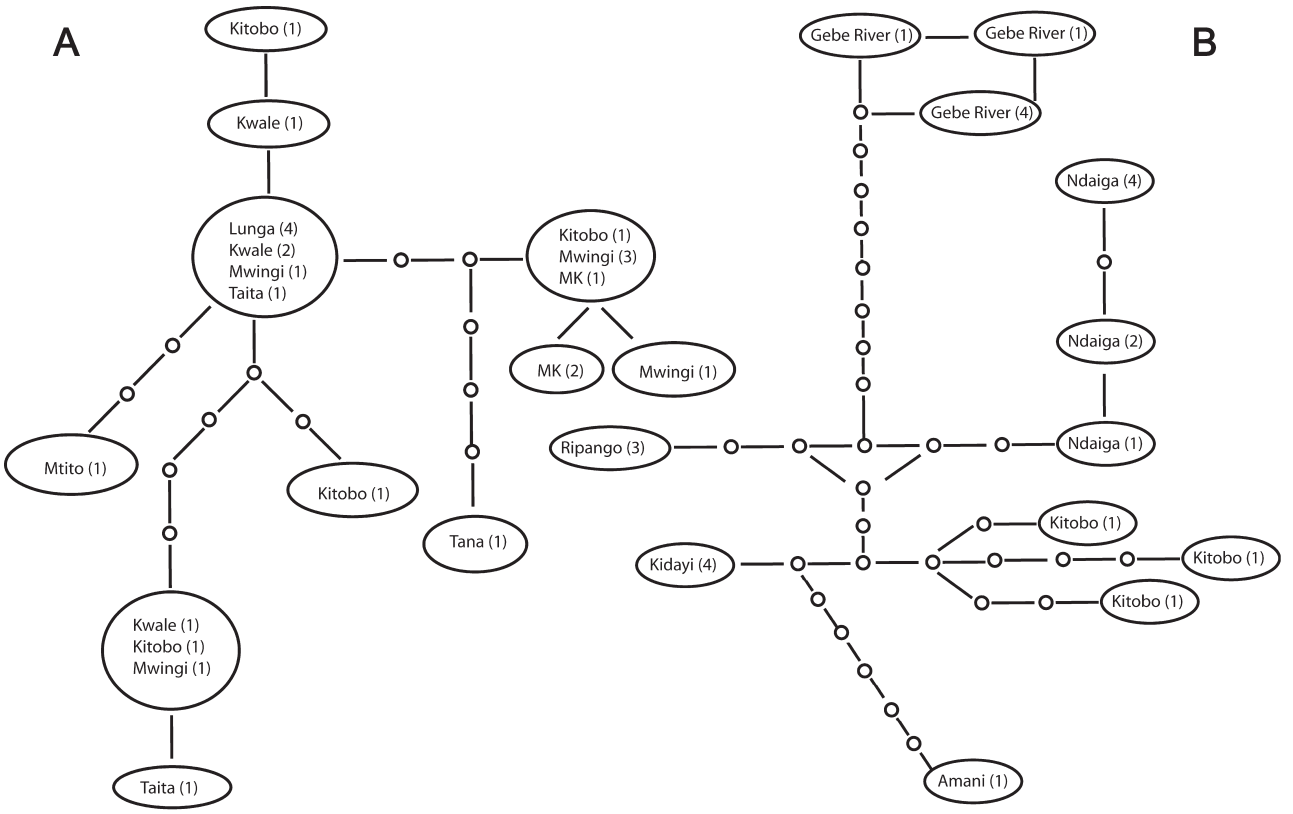

Figure 12
Haplotype networks of the clades of *Manga nubifera* (A. MN1, B. MN2).

melanodonta but sharper. However the small number of specimens examined does not permit to conclude whether this is a characteristic of that species or not.

The shape of the clasper and cucullus showed in *M. melanodonta* some slight differences between various areas, but it is unclear at present if more sampling in intermediate regions would not show a cline. Figure 14 shows for instance that in Uganda the clasper is rather slender and the cucullus clearly triangular, in Kenya the clasper is fairly similar but the cucullus more rounded and in Mozambique the clasper is wider and longer with a shorter cucullus. No such geographic variability was observed within *M. nubifera*.

The observations made during this study allow to better define the characteristics and species composition of the genus *Manga*. In fact, the examination of the types of *M. bisignata* Laporte 1973, described from Cameroun (Laporte 1973), showed that *M. bisignata* is wrongly placed here and represents a synonym of *Busseola quadrata* Bowden 1956 (**n. syn.**). Both specimens have the same habitus, and the male genitalia are identical in all respects (tegumen, valve, juxta, vinculum, aedeagus). The genus *Manga* presently includes then five species: *M. basilinea* Bowden 1956 (fig. 15), the type species of the genus, from West Africa, *M. belophora* Fletcher 1961 (fig. 16) from Ruwenzori and the three species collected in this study: *M. melanodonta*, previously known only from Uganda (Hampson 1910), *M. nubifera* known until now only from Congo forest (Hampson 1910), and *M. fuliginosa*, found only in Mozambique.

The molecular analysis is in agreement with the morphological observations. The three morphological species are clearly distinct at the molecular level. The three morphological types observed in the regional populations of *M. melanodonta* belong to three

Table 3. Infraspecific diversity (value ± SD) in *M. nubifera.*

Clade	MN1	MN2
Gene diversity	0.860±0.050	0.931±0.028
Nucleotide diversity	0.00439±0.00252	0.01025±0.0054
Pairwise differences	4.13±2.13	9.64±4.58

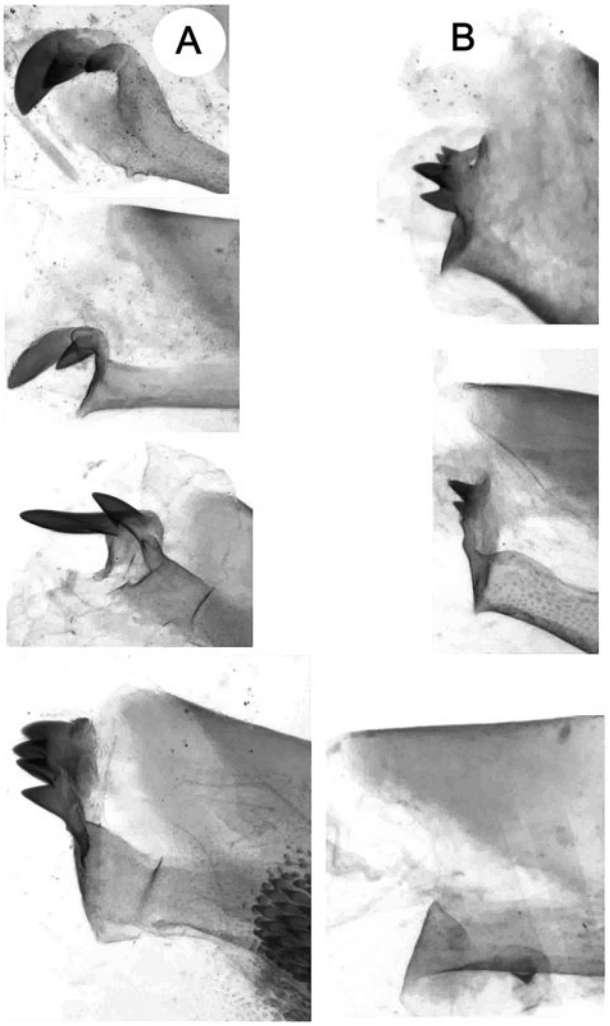

Figure 13
Variability in the shape and the number of spines of the sclerotized band of the aedeagus. A. *Manga melanodonta* (from up, specimens from Uganda, Mozambique, and two specimens from Kenya); B. *Manga nubifera*: three specimens from Kenya.

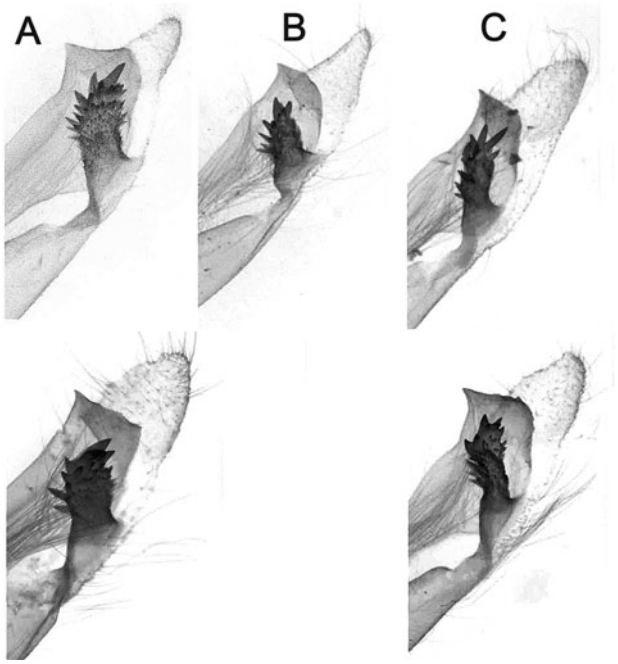

Figure 14
Variability in clasper and cucullus shape in *Manga melanodonta*. A. 2 specimens from Mozambique; B. 1 specimen from Uganda; C. 2 specimens from Kenya.

different clades, which suggests that they could be considered presently as sub-species, genetically isolated by distance. However the Uganda and Kenya clades are so close geographically that this isolation may vanish in the near future. The molecular data show moreover a similar fragmentation inside *M. nubifera* that was not detected morphologically, although the genetic isolation between both clades was apparently complete until very recently. They indicate also more recent diversification events that were not morphologically visible. With the exception of *M. fuliginosa*, for which only two males were collected from one locality, the molecular study revealed a complex evolutionary history of the group, with successive diversification events, migrations and colonizations of new areas.

The study of the intraspecific diversity of *M. nubifera* shed a light on the process of colonization during the recent past. It showed that the populations of Tanzania (Amani, Kidayi) seemed to have a different history from those of Mozambique (Ripango). The phylogenetic tree indicated that the population from Uganda (Ndaiga) was ancestral: the haplotype network suggests that the colonisation of the different areas occurred recently and that it followed different routes. The first one to Ethiopia (Gebe River) was probably due to a small population that was quickly isolated, favouring rapid genetic drift. A second route, eastern to Lake Victoria, enabled the colonization of Tanzania from which a population invaded South Kenya (Kitobo forest). The Kitobo forest is indeed a Tanzanian type forest, the north-eastern end of the Zambezian miombo ecological region (White 1986). Some excentred populations of Tanzania like Amani have likely small sizes and little gene flows with other populations and have a quicker rate of fixation. The Mozambique (Ripango) population seems to originate from a third route, western to Lake Victoria. It is genetically close to the Uganda population, in spite of a long geographic distance. This suggests that either

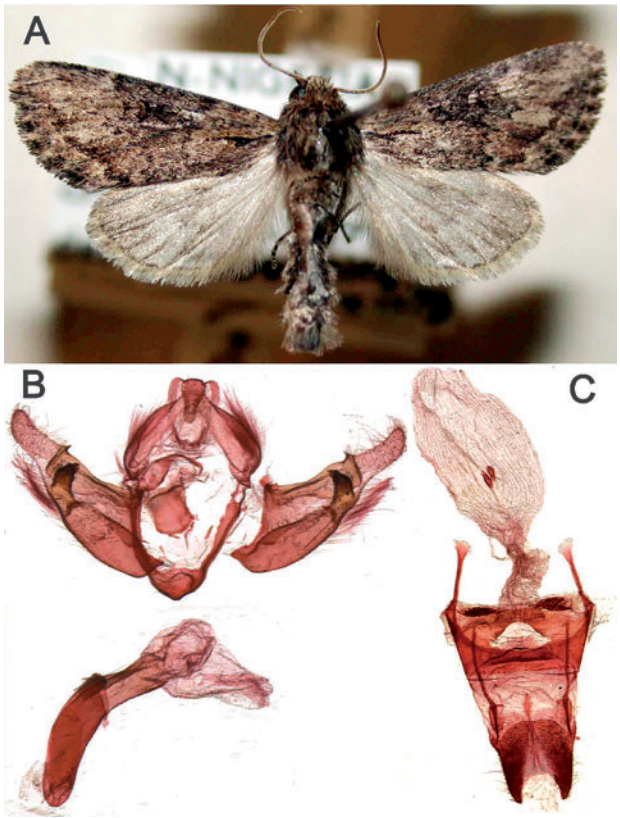

Figure 15
Manga basilinea. A. Male, type adult; B. Male genitalia; C. Female genitalia.

Figure 16
Manga belophora. A. Male, type adult; B. Male genitalia.

the colonization of Mozambique is very recent, or that the size of this population is high, reducing the genetic drift. Local populations were very homogeneous (only one haplotype for instance in Kidayi, or in Ripango, only 3 very close in Gebe River) except in Kenya and Uganda. This study showed also the influence of mountains in the isolation of populations. Indeed, the gene flow estimated by Slatkin's M value indicates that populations of south east and coastal Kenya can be considered as a single population when strong limitations to gene flow occur with the northern population. The probable cause of this isolation is not the geographic distance but the Machakos mountains that constitute a geographical barrier between both groups.

Independently of the morphological similarities, two facts suggest that the evolution of the group is recent. The first one is the impossibility of estimating the divergence dates using the method proposed by Gaunt & Miles (2002), because of the absence of variation of the slowly evolving sites they used. Indeed, the comparison by Gaunt and Miles of the pairwise difference (difference of 21 aminoacids among 477 for the whole cox1 gene) and the estimated date of divergence (about 100 million years) between the two moth species they used, *Manduca sexta* (L. 1764) (Sphingidae) and *Feltia jaculifera* (Guenée 1852) (Noctuidae), shows that only divergence events older than about 5 million years are likely to be dated (since, when considering a local molecular clock, about one aminoacid mutation occurred every 5 million years). For the part of the cox1 we sequenced (298 amino acids) a pairwise difference of 11 aminoacids was observed between both species. The evolutionary rate is then not very different from (and even a little slower) that of the whole gene (3.70% vs 4.40%), and recent divergence events could not be estimated. This result suggests however that the observed divergence events between *Manga* species are recent, and could have occurred in the last 5 million years. The second fact is the high host plant specialization of these insects, and more generally of the African noctuid stem borers, that is now demonstrated (Le Rü *et al.* 2006). Indeed, this specialization occurred on tropical monocot plants with C4 photsynthetic system. And it is known that C4 monocots were very rare before 9 Myr before present (BP) and became dominant and highly diversified between 9 and 4 Myr BP, when they replaced C3 plants (Jacobs *et al.* 1999). The borer specialization on these plants, that probably contributed to their diversification, could then only occur after the host plant diversification. The borer diversification may moreover have occurred rather a long time after that of host plants, resulting in sequential radiation such as observed in other insects *e.g.*

psyllids (Percy *et al.* 2004). The results show that such a case likely occurred for *M. melanodonta*, that colonized *Setaria* species recently, probably long after the plant diversification.

From these two facts it can be concluded that the evolution rate of the studied species is probably close to that estimated by Brower, since the calculated divergence dates were less than 5 million years. Moreover, the comparison of the estimated divergence dates with the main paleo-climatic events that occurred in Africa in the past five million years enables to confirm this rate. Indeed, in addition to the part played in divergence by the host plant specialization, the influence of major paleo-climatic events (dry and cold periods that favoured speciation processes by isolation in refuges followed by humid and warm periods favouring dispersion), that occurred simultaneously in vast regions, is suggested by the similar genetic distance observed between infraspecific clades. The comparison of the estimated divergence dates with the occurrence of these major events shows that both match, which enables to propose the following scenario for the evolution of the group.

1. The ancestor of the group lived in austral Africa in stems of *P. maximum*. A first fragmentation event occurred about 4.6 Myr BP, which resulted in the species *M. fuliginosa* and the ancestor of *M. melanodonta* and *M. nubifera.* An important environmental event occurred at that time in this region (Lovett 1993): the reinforcement of the Benguela current that occurred around 5 Myr BP, resulting in a greater aridity in south western Africa and a compression of the southern Guineo-congolian forests. These conditions were favourable for speciation and then it can be supposed that both events are related.
2. From this time until about 3.5 Myr BP the African climate was humid (Lovett 1993; De Menocal 1995), favouring colonization of new areas. The ancestor of *M. melanodonta-M. nubifera* probably extended towards the northeast. Maybe the gene flow between the northern population, that later resulted in *M. nubifera*, and the southern, which resulted in *M. melanodonta*, reduced progressively. The divergence in environmental preferendums between both populations (dry forests and one host plant for *M. nubifera*, humid forests and several host plants for *M. melanodonta*) may have begun then or during the fragmentation event that occurred next.
3. The fragmentation between both species occurred around 3 Myr BP. This was the first result of several successive cycles of dry-humid periods that occurred between 3.4 and 1.7 Myr BP. This period was very favourable to speciation in other groups such as mammals (Bobe & Behrensmeyer 2004), which showed a high species turn-over. Particular dry and cold periods occurred around 2.5 Myr BP (Bonnefille 1983) and 1.7 Myr BP (De Menocal 1995). For *Manga* species, south-north migrations were no longer possible at this time, resulting in the divergence of both species. The colonization by *M. melanodonta* of new host plants belonging to *Setaria*, if not yet done, may have occurred when the species was limited to altitudinal forested refuges during the dry periods.
4. In spite of these successive cycles no other still visible fragmentation event occurred in *Manga* during about 1 Myr, maybe because of the shortness of the cycles (around 200 thousand years). On the contrary, this period was probably favourable to the colonization of new areas: it can be assumed that *M. nubifera* colonized both sides of the Rift Valley, with some isolation by distance beginning, and that *M. melanodonta* began to migrate northwards. Humid periods favoured apparently much more the expansion of the species adapted to humid environment, thanks particularly to the specialization on *Setaria*.
5. At about 2 Myr BP, a new fragmentation occurred in both species, resulting in the two clades of *M. nubifera*, and the three clades (Mozambique, Kenya, Uganda) of *M. melanodonta*.
6. Around 1 Myr BP, a new strong dry period occurred (De Menocal 1995), which likely resulted in the fragmentation of the Uganda and Kenya clades of *M. melanodonta*. Apparently, it had no influence on *M. nubifera* diversification.
7. From this time, the western clade of *M. nubifera*, as shown by the haplotype network, expanded highly, with the colonization of new areas in many directions: northern to Ethiopia and southern to Tanzania and Mozambique, and the invasion of South Kenya from Tanzania. Most populations of *M. melanodonta* seem to have remained isolated in different regions, but an expansion also occurred in the Kenyan clade west of the Rift Valley, since one individual was found in Taita. This expansion was apparently much more limited than for *M. nubifera*, which would indicate that the conditions of the last million years, with the successive glacial cycles, were not suitable for strong migrations of the species adapted to humid areas, *M. melanodonta*.

In addition to the influence of paleo-climatic events, geological barriers played a part in this evolution. The role of the Rift Valley is thus particularly visible at two levels. First, it explains the isolation of both clades of *M. nubifera* for about 2 Myr (the colonization of the east of the Rift Valley in South Kenya from Tanzania is very recent) and their different expansion. The clade east of the Rift could apparently not expand, when the western clade invaded many new areas. Second, in *M. melanodonta*, the genetic distance between the two clades east and west of the Rift (KT and RC) is higher than the one between the two clades of Uganda. Both fragmentation events are probably due to the same dry period at 1 Myr, but the two Kenyan populations were at that time likely already genetically distant because of previous stronger isolation by the Rift.

Probably of less importance, but favouring local diversification at least temporarily, is the influence of mountains, such as was observed for the Machakos mountains east of the Rift Valley.

From this first study on the evolution of a Noctuid stem borer genus, it can be concluded that the combination of three forces has shaped the diversification of species in the past million years. Most divergence events resulted from paleo-climatic changes, but geological barriers and adaptation to new host plants played also significant parts by themselves or by enhancing the climate effects.

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